Environmental Health Perspectives
Vol. 23, pp. 51–61, 1978

Studies on the Microsomal Metabolism and Binding of Polybrominated Biphenyls (PBBs)

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The metabolism of polybrominated biphenyls (PBBs) was studied *in vitro* by using rat liver microsomes in the presence of NADPH and atmospheric O_2. Quantitative recoveries of all PBBs were obtained after incubations with control or 3-methylcholanthrene (MC) induced microsomes. Of the twelve major components, losses of only peaks 1 (2,4,5,2′,5′-pentabromobiphenyl) and 3 (a hexabromobiphenyl) were observed following incubations with microsomes from phenobarbital (PB)- or PBBs- pretreated rats. Of seven structurally identified PBB components, only peak 1 has a bromine-free *para* position. Peaks 1, 2, and 5 all have two adjacent unsubstituted carbons, yet only peak 1 is metabolized. Of two dibromobiphenyl model compounds studied, the 2,2′-congener was very rapidly metabolized by PB-induced microsomes whereas its 4,4′-isomer was not. These results suggest that the presence of a free *para* position is required for the metabolism of brominated biphenyls. Of lesser importance appears to be the number of bromines or the availability of two adjacent unsubstituted carbons. *In vivo* evidence for the metabolism of peaks 1 and 3 was also provided by their drastically diminished levels in liver and milk extracts. When a ^{14}C-PBB mixture consisting almost exclusively of peaks 4 (2,4,5,2′,4′,5′-hexabromobiphenyl) and 8 (2,3,4,5,2′4′,5′-heptabromobiphenyl) was incubated with PB- or PBBs- induced microsomes and NADPH, only traces of radioactivity remained with the microsomes after extensive extraction. However, less radioactivity was bound to microsomes from MC pretreated or especially control rats. No radioactivity was bound to exogenous DNA included in similar microsomal incubations, regardless of the type of microsomes used. Under the same conditions, [^{3}H]-benzo[a]pyrene metabolites were bound to DNA, and PBB-induced microsomes enhanced this binding more than six-fold.

Introduction

Polybrominated biphenyls (PBBs), fire retardants closely related to the polychlorinated biphenyls (PCBs), were mistakenly mixed with cattle feed in Michigan in 1973 (*1*). Contaminated meat and dairy products made their way to the tables of Michigan residents due to a lag of several months in the discovery of the contamination (*1*). The economic and health effects due to this incident have been reviewed by Dunkel (*2*), Mercer et al. (*3*), and Kay (*4*). More recently traces of PBBs were detected in soil, plants, water, fish, and human hair in both New York and New Jersey (*5*).

PBBs have been shown to be potent inducers of hepatic and kidney microsomal drug metabolizing enzymes. They cause a mixed-type induction similar to that caused by treatment with both phenobarbital (PB) and 3-methylcholanthrene (MC) (*6–8*). The two major components of PBBs, 2,4,5,2′,-4′,5′-hexa- and 2,3,4,5,2′,4′,5′-heptabromobiphenyls (peaks 4 and 8, respectively) have been shown to be strictly PB-type inducers (*9, 10*). The effects of other PBB components on drug metabolizing enzymes are not known. Also unknown is the metabolic fate of PBBs, even though at least some of them may be metabolized by hepatic mixed-function oxidases in a manner similar to that for PCBs.

Since PBBs are highly persistent in the body, and since little is known about their biochemical fate, it was necessary to investigate their susceptibility to biotransformation. For this purpose, an *in vitro*

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system, utilizing microsomes isolated from rats pretreated with various agents, was used to study the effect of bromine content and structure of the PBB components on their susceptibility to metabolism by the different types of microsomal mixed-function oxidases. Evidence is also shown for the *in vivo* metabolism of PBBs. Studies are also reported on the ability of liver microsomes to convert PBBs to metabolites capable of binding to microsomal macromolecules or exogenous DNA.

Methods

Chemicals

The PBBs sample used in this study was obtained from a feed mixing mill shortly after the discovery of its accidental mixing into cattle feed. It is of the FireMaster type manufactured by Michigan Chemical Corp., St. Louis, Mich. The gas chromatographic profile is shown in Figure 1. Also included

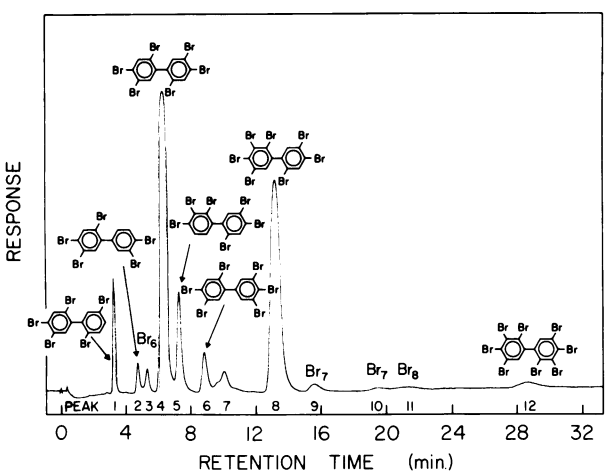

FIGURE 1. Gas chromatographic profile of FireMaster PBBs and structures of identified components. The unidentified components are designated with their bromine content. All twelve main components are numbered according to their gas chromatographic chronological order.

in this figure is the numbering system used to identify the components of the mixture. The structures and bromine contents are shown for those components which have been identified (*11, 12*). Peak 4 is the major component of the mixture (56% by weight), previously identified as 2,2',4,4',5,5'-hexabromobiphenyl (*13, 14*). The column packing for the GLC was 3% OV-1 on Gas Chrom Q, 100-120 mesh from Supelco, Inc., Bellefonte, PA. ^{14}C-PBB with a specific activity of 9.34 mCi/mmole was purchased from New England Nuclear, Boston, Mass. The gas chromatographic profile of this material is shown in Figure 2.

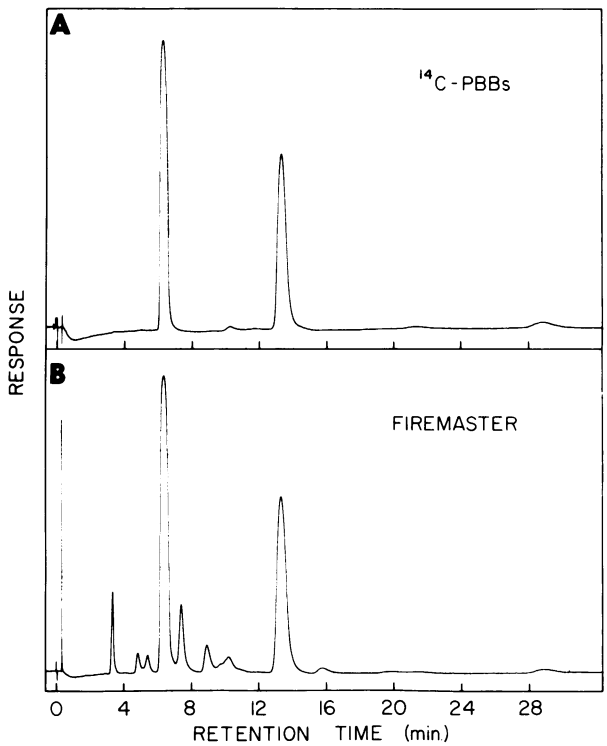

FIGURE 2. Gas chromatographic profiles of (*A*) ^{14}C-PBBs and (*B*) the FireMaster PBBs mixture.

Phenobarbital sodium U.S.P. was purchased from Merck and Co., Inc., Rahway, N. J. Polyethylene glycol (PEG, approximate molecular weight 400), 3-methylcholanthrene, NADP$^+$, sodium dodecyl sulfate, Florisil (magnesium silicate activated at 1250°F), DNA (from salmon, type III), benzo[a]pyrene, butylated hydroxytoluene, D,L-trisodium isocitrate (type I), and isocitrate dehydrogenase (from pig heart, type IV, highly purified) were all obtained from Sigma Chemical Co., St. Louis, Mo. [6-^{3}H]-Benzo[a]pyrene (batch 4) was purchased from Amersham/Searle Corp., Arlington Heights, Ill. and was used without further purification. Specific activity was 29 Ci/mmole. Dimethyl-POPOP (1,4-bis [2-(4-methyl-5-phenyloxazoly)] benzene), PPO(2,5-diphenyloxazole), and Triton X-100 were all scintillation grade, purchased from Research Products International Corp., Elk Grove, Ill. Ethyl acetate, glass distilled, suitable for pesticide analysis, was obtained from Burdick and Jackson Laboratories Inc., Muskegon, Mich. Pitocin (oxytocin) was obtained from Parke-Davis & Co., Detroit, Mich. Sodium pentobarbital (Nembutal) was purchased from Abbott Laboratories,

North Chicago, Ill. All other chemicals were of reagent grade quality.

Animals and Treatments

Male rats of the Sprague-Dawley strain were from Spartan Research Animals, Haslett, Mich. Control rats, weighing 250–275 g, were not given any treatment before sacrifice. PBBs-pretreated rats were between 150 and 175 g when injected, IP, with 90 mg PBBs/kg (in 2 ml PEG/kg) 1 week before sacrifice. Rats treated with PB weighed 225–250 g and were given four daily IP injections at 50 mg/kg (in 1 ml H_2O/kg) and killed on the fifth day. Rats given MC ranged between 75 and 100 g and were injected IP, with 20 mg MC/kg in 2 ml PEG/kg 36 and 24 hr before sacrifice. Feed and water were available to all animals *ad libitum* except for the night before sacrifice, when only water was accessible.

Isolation of Microsomes

Microsomes were isolated according to previously described procedures (*15, 16*). Rats were sacrificed by decapitation, and their liver microsomes were isolated and then washed with a 0.3*M* sucrose containing 0.1*M* Na pyrophosphate to remove ribosomes and adsorbed-proteins. The microsomes were stored at −20°C in 50*mM* Tris-HCl, pH 7.5, containing 50% glycerol and 0.01% butylated hydroxytoluene.

Collection of Rat's Milk and Its Extraction for PBBs

One day after parturition, lactating rats weighing about 300 g were injected, IP, either with 90 mg PBBs/kg (in 1.5 ml corn oil/kg) or with the equivalent amount of corn oil. Milk was obtained on the afternoons of days 2, 4, 6, 7, and 9 after the injections. The samples from each dam were pooled together for later extraction. The dams were separated from their litters 2–3 hr before the milking process. Milk ejection was stimulated with a 0.15 ml IP injection of oxytocin (Pitocin), 10 U/ml. After 5 min, the rat was lightly anesthesized by injecting 0.1–0.15 ml of 60 mg/ml sodium pentobarbital. Milk was collected by gentle suction by use of a simple hand-made suction apparatus employing a water aspirator. Solvent extractions and acetonitrile partitioning of PBBs in the milk were done according to the Pesticide Analytical Manual procedure for extraction of organochlorine residues from milk (*17*) which was scaled down for samples of 2–3 ml. The extracts were run over a small Florisil column and analyzed for PBBs by a Hewlett-Packard 402 gas chromatograph. The gas chromatograph was equipped with a pulsed-^{63} Ni electron-capture detector fitted with a 6-ft glass column and operated at 270°C. An Argon-methane (95:5) mixture was used both as a carrier and purge gas.

In Vitro Microsomal Metabolism of PBBs

The NADPH-dependent metabolism of PBBs was studied by assaying for their time-dependent disappearance when incubated with liver microsomes. Microsomal incubations (5ml) were carried out aerobically at 37°C in 50*mM* Tris-HCl buffer, pH 7.5, in the presence of an NADPH generating system containing 25 nmole $MnCl_2$, 25 μmole $MgCl_2$, 2.5 μmole $NADP^+$, 20 μmole D,L-isocitrate, and 0.5 units of isocitrate dehydrogenase. The microsomes were added at 1 mg protein/ml. The following amounts of substrates were delivered in 5 μl PEG: 10 μg PBBs and 50 μg of either 2,2′- or 4,4′-dibromobiphenyls. Incubations were carried out in a Dubnoff shaker, and ethyl acetate extractions were performed at the end of each incubation period, as well as for the 0 time, non-incubated samples. The extracts were water washed and the organic solvent dried over sodium sulfate before evaporation to about 1 ml. The concentrated extract was then run on a small Florisil column to remove undesirable polar material and concentrated to a suitable volume before GLC analysis. For the analysis of the dibromobiphenyls the column temperature was reduced to 200°C. The recoveries of the different bromobiphenyls were determined by cutting and weighing their corresponding gas chromatographic peaks. Since the gas chromatographic detector responses for peaks 1 and 3 are not known, the response to pure peak 4 was used to quantitate these congeners, assuming all three responses to be identical. All experiments were run in duplicate and the difference between the means of 0-time and *t*-time recoveries for each peak was statistically analyzed by the student's *t*-test, $p < 0.05$. The PBBs present in the liver microsomes from PBBs pretreated rats were extracted and analyzed by the same procedure.

Covalent Binding of PBBs to Microsomal Macromolecules

Incubation conditions used to determine the extent of binding to macromolecules were nearly identical to those used to assay for PBB metabolism. Incubation mixtures contained 0.5 mmole Tris-HCl, pH 7.5, 50 μmole $MgCl_2$, 50 μmole

$NADP^+$, 40 μmole D,L-isocitrate, 50 nmole $MnCl_2$, 0.5 units of isocitrate dehydrogenase, and approximately 80 nmole ^{14}C-PBBs (50 μg), and 10 mg of control, MC, PB-, or PBB-induced microsomal protein in a final volume of 10 ml. Control incubations were identical except that $NADP^+$ was omitted. Reactions were initiated by the addition of substrate (PBBs in 50 μl acetone). Reaction vessels (50 ml glass centrifuge tubes) were vortexed, then placed in a Dubnoff metabolic shaker for 1 hr at 37°C. Reactions were terminated by the addition of 20 ml ethanol. The turbid suspensions were vortexed, then centrifuged. Supernatants were removed, and ten 10 ml ethanol extractions were made. The ethanol was vigorously pipetted into the centrifuge tubes to help dissociate the pellets, and the tubes were vortexed each time. Little if any radioactivity was removed by the last four extractions. When the last ethanol extractions had been made, the macromolecular residues were transferred to scintillation vials for radioactivity analysis.

Binding of ^{14}C-PBBs and ^{3}H-Benzo[a]pyrene to DNA

For the determination of binding to DNA, 20 mg of DNA in water replaced an equal volume of Tris buffer. Reactions were initiated by the addition of substrates at the following final concentrations: 8 *μM* ^{14}C-PBBs (in 50 μl acetone) and 80 *μM* ^{3}H-benzo[a]pyrene (in 500 μl acetone). Incubation reactions containing DNA were terminated by the addition of 10 ml water–saturated phenol and 0.5 ml of 10% (w/v) sodium dodecyl sulfate. Following centrifugation, the upper (aqueous) phases were transferred to clean 50 ml clinical centrifuge tubes. After adding 0.5 ml of 2*N* NaCl, 20 ml of ethanol was added, and the tubes were centrifuged. Pellets were redissolved in 4.5 ml of 50*mM* Tris-HCl, pH 7.5, and incubated at 37°C for 15 min. After adding 0.5 ml of 2*N* NaCl, 10 ml of ethanol was added to reprecipitate the DNA, and the tubes were centrifuged. This cycle was performed four times, and little if any radioactivity was found in the last two washes. The final DNA pellets were transferred to scintillation vials to determine the amount of bound radioactivity.

Results

In Vitro Metabolism of PBBs by Different Types of Microsomes

The individual components of PBBs were investigated for their susceptibility to NADPH-dependent biotransformation by rat liver microsomal enzymes. Microsomes from PB- or PBBs-pretreated rats caused preferential losses of both peaks 1 and 3 (penta- and hexabromobiphenyls, respectively) from the mixture in a time-dependent manner (Figs. 3*A* and 3*B*). The maximum rate of metabolism of peak 3 occurred during the first 15 min. where it reached 0.9 pmole/min-mg protein. The maximum rate of peak 1 metabolism occurred during the second 15 min period. However, its rate of metabolism (1.6 pmole/min-mg protein) was greater than the maximum rate seen for peak 3. Microsomes from PBBs- pretreated rats metabolized these components at rates of 0.9 and 2.4 pmole/min-mg protein during the same time periods. When an incomplete NADPH-generating system (-NADP) was used, all peaks were recovered almost quantitatively after 1 hr of incubation with PB-induced microsomes (Table 1). Microsomes from control or MC-pretreated rats were unable to significantly affect the recovery of any component of the mixture (Figs. 3*C* and 3*D*).

Metabolism of Dibromobiphenyls by PB-Microsomes

When two model PBB congeners, 2,2′- and 4,4′-dibromobiphenyls, were incubated with liver microsomes from PB-treated rats, the 4,4′-congener was recovered quantitatively (Fig. 4). The 2,2′-congener was metabolized at a very rapid rate; about 160 nmole had disappeared by the time the first time-point was taken (15 min).

Covalent Binding of ^{14}C-PBB to Macromolecules

The covalent binding of ^{14}C-PBB to microsomal macromolecules was extremely small, less than 0.05% of the substrate was bound (Table 2). However, the extent of binding was dependent upon the type of microsomes used to activate the compounds. Microsomes isolated from animals pretreated with PB or PBBs bound five times the amount of radioactivity as was bound by control microsomes. Induction of MC more than doubled the amount of binding.

Denatured exogenous DNA was included in another set of *in vitro* microsomal incubations in order to determine whether PBBs or their metabolites could covalently bind to it. Positive results would indicate that these ^{14}C-PBB components are potentially mutagenic and carcinogenic. Parallel incubations were also carried out by use of ^{3}H-benzo[a]pyrene in place of ^{14}C-PBBs. Benzo[a]pyrene is carcinogenic when metabolically activated, and

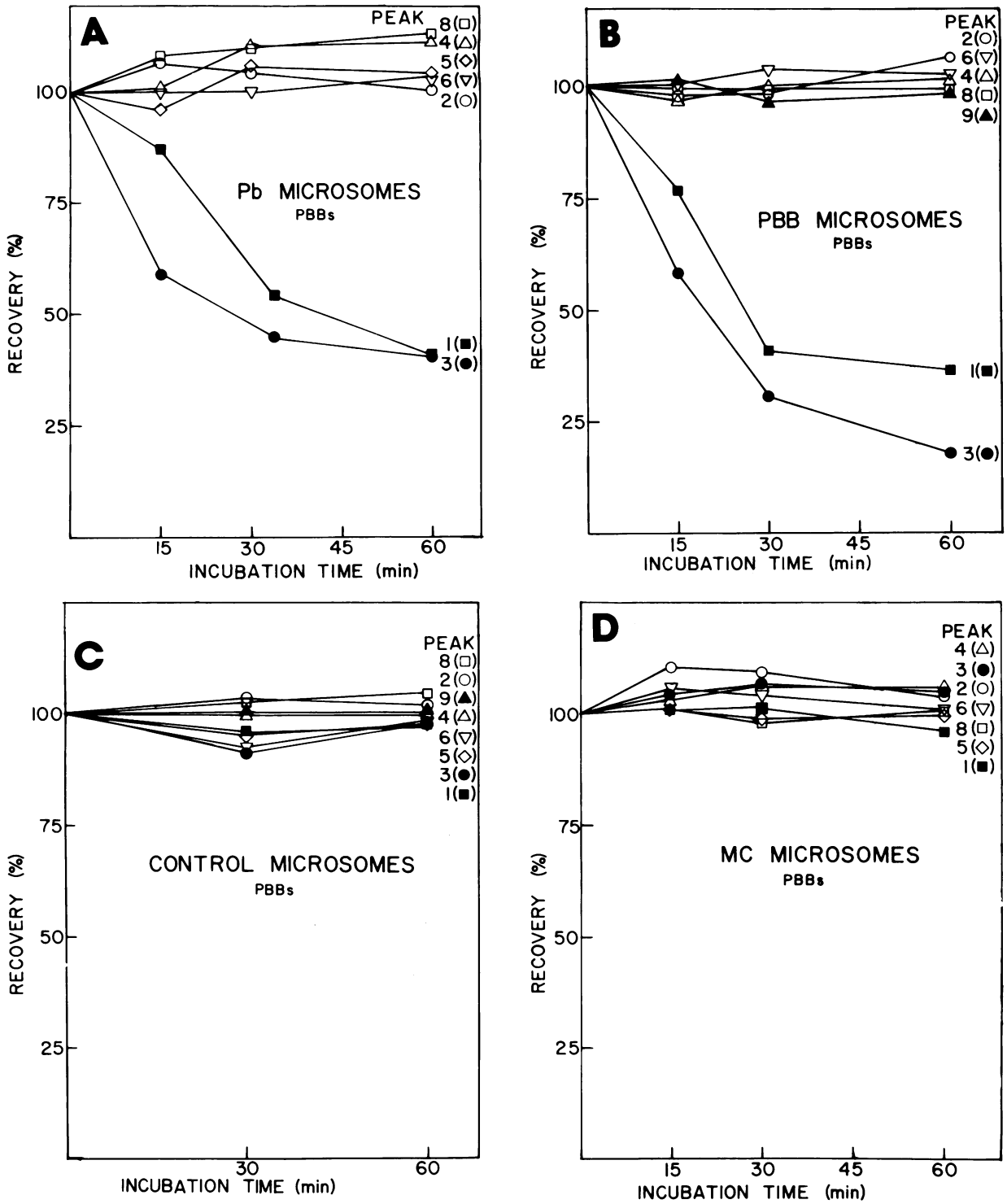

FIGURE 3. Time course for the metabolism of PBBs by liver microsomes from (*A*) PB-, (*B*) PBB-, (*C*) control , or (*D*) MC- pretreated rats. Aerobic incubations for different times were carried out separately in 5 ml total volume. Each incubation mixture contained the NADPH-generating components described under Materials and Methods, 5 mg liver microsomal protein and 10 μg PBBs delivered in 5 μl PEG. PBBs extractions and assays were performed as described in Materials and Methods. Each point represents an average of two determinations. Peaks 1 and 3 at all time points in presence of (*A*) PB- or (*B*) PBBs- induced microsomes were statistically different from those at zero time ($p < 0.05$). All other peaks at all time-points were not statistically different from their corresponding values at zero time except for peak 8 at 60 min in *A* ($p < 0.05$).

Table 1. Recoveries of PBBs following incubations with PB-induced microsomes.[a]

Peak	+NADPH	−NADPH
1	41[b]	88
2	101	114
3	41[b]	94
4	111	95
5	104	95
6	104	106
7	104	105
8	113[b]	96
9	124	90
10	96	100
11	98	109
12	125	103

[a] A10-μg portion of FireMaster PBBs was aerobically incubated with 5 mg protein of PB-induced rat liver microsomes for 1 hr at 37°C in the presence and absence of NADPH. PBBs extractions and assays were performed as described in Materials and Methods. The GC profile of PBBs of zero time incubation was identical to that of FireMaster PBBs, and the recoveries of all zero time peaks were > 85%. Each value is an average of two determinations and represents the per cent recovery after one hour incubation as compared to the corresponding value at zero time. The average SEM in per cent recovery for the control incubations (−NADPH) were 6.6 and 3.4 for the zero time and 1 hr incubations, respectively. For the incubations with NADPH, they were 1.6 and 3.0 for zero time and 1 hr, respectively.

[b] Significantly different from those at zero time ($p < 0.05$).

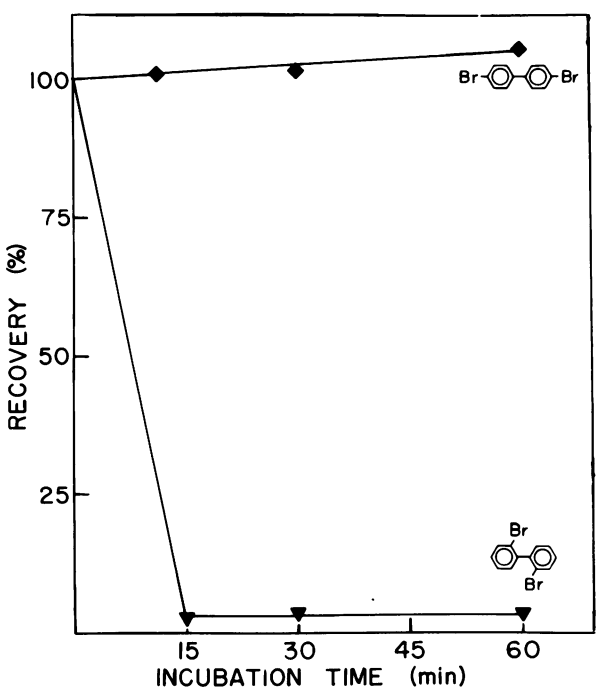

FIGURE 4. Time course for the metabolism of two dibromobiphenyl model compounds by PB-induced microsomes. Experimental conditions were similar to those described for Fig. 3 except that each of the two dibromobiphenyls was added to a final concentration of 34 μM.

Table 2. Effects of microsomal enzyme induction on the *in vitro* NADPH-dependent covalent binding of ^{14}C-PBB metabolites to microsomal macromolecules.[a]

Types of microsomes	Quantity of PBBs bound		Rate of binding, fmole/min-mg protein
	ng	% of substrate	
Control	4.3	0.0086	11
MC	10	0.021	27
PB	22	0.044	58
PBBs	22	0.044	59

[a] A 50-μg portion of ^{14}C-PBBs were aerobically incubated with 10 mg of rat liver microsomal protein for 1 hr at 37°C in the presence of NADPH. Reaction mixtures were then exhaustively extracted with ethanol to remove unbound substrate and metabolites, and the amount of radioactivity remaining associated with the microsomal macromolecules was determined. Microsomes were isolated from control rats, or from rats pretreated with MC, PB, or PBBs. When incubations were carried out in the absence of NADPH, approximately 25 ng of PBBs remained associated with the macromolecules, regardless of the type of microsomes used.

its metabolites are known to bind covalently to DNA (*18*). The experiments with benzo[a]pyrene served both as a positive control for the ^{14}C-PBB binding experiments and determined the effect of PBBs induction of microsomal enzymes on the DNA binding of a secondary agent. The results are shown in Table 3.

No radioactivity could be detected on DNA following incubation with ^{14}C-PBBs. The type of microsomes used or the presence or absence of NADPH in the incubation mixture made no difference. The data in Table 3 for PBBs represents the limit of sensitivity of the assay, and were calculated assuming that 10 cpm above background had been observed. The samples were all below this level of radioactivity. When ^{3}H-benzo[a]pyrene was substituted for ^{14}C-PBBs, significant quantities of radioactivity were found to be bound to DNA. Microsomes from PB-pretreated animals were no better than control microsomes in their ability to catalyze binding, while PBB and MC induction greatly increased the microsome-catalyzed covalent binding of benzo[a]pyrene metabolites to DNA.

Discussion

FireMaster is a complex mixture of polybrominated biphenyls. The major components of the mixture can be separated and assayed by GLC. The *in vitro* metabolism of the individual components of the mixture can therefore be studied by gas chromatographic analysis of the components remaining after incubation with liver microsomes. There was no noticeable losses of any of the PBBs upon incubation with control microsomes and NADPH. This observation agrees with the inability of microsomes

Table 3. Effects of microsomal enzyme induction on the *in vitro* NADPH-dependent covalent binding of ^{14}C-PBB and ^{3}H-benzo[a]pyrene metabolites to DNA.[a]

Substrate	Type of microsomes	Quantity of substrate bound			
		ng	Fraction of substrate	Residues/10^7 bases	Rate of binding, fmole/min-mg protein
^{14}C-PBBs	All	<0.3	$<7 \times 10^{-6}$	<.11	<0.9
^{3}H-benzo[a]pyrene	Control	5.5	27×10^{-6}	3.7	36
	MC	86	430×10^{-6}	58	570
	PB	5.1	25×10^{-6}	3.4	33
	PBBs	33	160×10^{-6}	22	220

[a] A 50-μg (80 nmole) portion of ^{14}C-PBBs or 200 μg (300 nmole) of ^{3}H-benzo[a]pyrene was aerobically incubated with 10 mg of rat liver microsomal protein for 1 hr at 37°C in the presence of NADPH and 20 mg of denatured DNA. DNA was then isolated and exhaustively extracted with ethanol to remove unbound substrates and metabolites, and the amounts of radioactivity remaining associated with the DNA were determined. Microsomes were isolated from control rats, or from rats pretreated with MC, PB, or PBBs. When incubations were carried out in the absence of NADPH, approximately 1 ng of benzo[a]pyrene metabolites were bound to the DNA, regardless of the type of microsomes used. Results with ^{14}C-PBBs were independent of NADPH.

from control rats to metabolize certain PCBs that were metabolized by PB-induced microsomes (*19*). When incubated with microsomes from PB- or PBBs-pretreated rats, two of the twelve major PBB components were metabolized. These two components, peaks 1 (2,4,5,2′,5′-pentabromobiphenyl) and 3 (a hexabromobiphenyl of unknown structure), are estimated to be present at approximately 2 and 1% of the total mixture, respectively. Of the mixture, peak 3 was metabolized (by PB-induced microsomes) at 0.9 pmole/min-mg protein, and its maximum rate of metabolism occurred during the first 15 min of incubation. Peak 1, however, was metabolized at a faster rate (1.6 pmole/min-mg protein) but the maximum rate was seen during the second 15 min incubation period. These preliminary results suggest that, even though the maximum rate of metabolism of peak 3 is less than the maximum rate of metabolism of peak 1, it is the preferred substrate, presumably for the same enzyme, and therefore is metabolized first. As the concentration of peak 3 decreases, the rate of peak 1 metabolism increases. The fact that peak 1 was metabolized at a higher maximum rate than peak 3 (1.6 vs. 0.9 pmole/min-mg protein, respectively) could also be due to the presence of more of peak 1 than of peak 3 in the mixture (approximately 2 and 1%, respectively). The requirement for NADPH suggests the involvement of the microsomal monooxygenase system containing cytochrome P-450. It also appears that the microsomal monooxygenase system involved in the metabolism of certain components of PBBs is the type induced by PB. The metabolism of the same two components (peaks 1 and 3) by microsomes from PBBs-pretreated rats was similar to that of PB-induced microsomes. Even though PBBs are known to cause a mixed-type induction (*6–10*), microsomes from MC-pretreated rats seemed incapable of metabolizing any of the major components of PBBs.

Our results seem to indicate that the bromine content of the molecule is not as important in determining metabolism as is the distribution of bromines on the biphenyl nucleus. The metabolism of peak 1 (2,4,5,2′,5′-pentabromobiphenyl) seems to agree with the metabolism of the analogous pentachlorinated molecule into hydroxylated derivatives *in vitro* (*19*) as well as *in vivo* (*20*). Peak 2, the second pentabrominated component of PBBs, with bromines at the 2,4,5,3′, and 4′ positions was found to be resistant to metabolism. The same pentachlorinated biphenyl homologe was found to be persistent in tissues from humans (*21*) as well as chickens (*22*). Peaks 3 through 6 are all hexabrominated biphenyls, but only peak 3 is metabolized. Unfortunately, at this time the structure of peak 3 is not known, but all those PBBs whose structure is known so far have a bromine at each of the *para* positions, except peak 1, which has one bromine-free *para* position. While each of peaks 1, 2, and 5 has two adjacent unsubstituted carbons, only peak 1 (of the structurally identified congeners) is metabolized by the microsomal monooxygenase system. These results seem to agree with the persistence in human tissue (*21*) of 2,4,5,2′,4′,5′- and 2,3,4,2′,4′,5′-hexachlorobiphenyls (the two equivalents of peaks 4 and 5 in PBBs, respectively) and the apparent resistance to *in vivo* metabolism of all those PCBs with a 4,4′-substitution in avians (*22, 23*). Therefore, it appears that the presence of two adjacent unsubstituted carbons is not sufficient by itself to render the brominated biphenyl molecules vulnerable to microsomal oxidation. Bromination of both *para* positions seems to render the PBB molecule resistant to microsomal metabolism regardless of the number of bromines or their dis-

tribution on the biphenyl nucleus, or the presence of two adjacent unsubstituted carbons.

Of the two model dibromobiphenyls studied, the 2,2′-dibromobiphenyl was rapidly metabolized while the 4,4′-congener was not. This lends further support for the necessity of a free *para* position to render the halogenated biphenyl susceptible to metabolism. The rapid metabolism of 2,2′-dibromobiphenyl agrees with the results on the rapid metabolism of 2,2′-dichlorobiphenyl by microsomes from both PB-pretreated female rats (*24*) and male rabbits (*25*). In the first study, there was a reduction in the metabolism and diversity of the hydroxylated metabolites when 2,4′-dichlorobiphenyl was used instead of the 2,2′-isomer (*24*).

Data were obtained to correlate *in vitro* metabolism with *in vivo* tissue levels of the various PBBs. Even though the distribution of PBBs in rat milk (Figs. 5*C* and 5*D*) and male rat liver microsomes (Figs. 5*E* and 5*F*) are not the same, they both are significantly changed from standard PBBs (Figs. 5*A* and 5*B*). Most noticeable is the preferential reduction in peaks 1 and 3 in rat liver microsomes and to a lesser extent in rat milk, especially when both components are compared to peak 2. It is also worth noting that the hepta- (peaks 8-10) and octa- (peaks 11 and 12) brominated biphenyls are reduced in the milk as well as peaks 8 and 10 in the liver. Furthermore, peak 6 (a hexabrominated component) is preferentially enriched in the hepatic tissue. These observations probably reflect differences in the distribution of the PBB components among different animal tissues rather than to their metabolic conversion *per se*.

The metabolism of PBBs is especially important, since one likely mechanism for the expression of their toxicity could be the metabolic activation by one or more of the microsomal cytochrome P-450 hemoproteins into epoxides. Many epoxides are reactive electrophiles, which can covalently bind to protein, RNA, and DNA. The binding of chemicals to DNA can lead to mutations and cancer. Because of the demonstrated carcinogenicity of PCB mixtures, in mice (*26, 27*) and rats (*28*), and because 4-chlorobiphenyl and PCBs can covalently bind to macromolecules (*29, 30*), it was of interest to determine whether PBBs could be activated into metabolites capable of covalently binding to protein and more importantly DNA. And, although it appears to be absent from FireMaster, the possibility

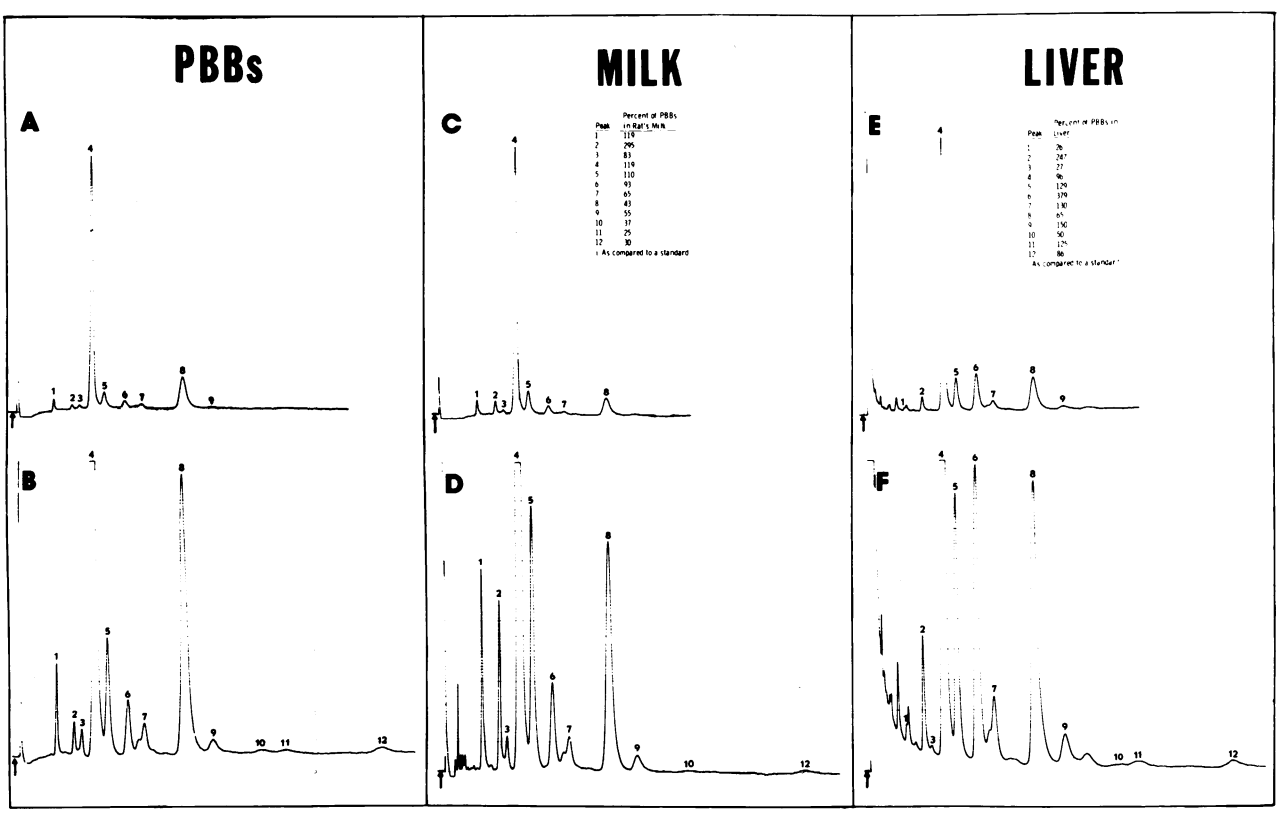

FIGURE 5. The distribution of PBBs in (*A, B*) FireMaster, (*C, D*) milk, and (*E, F*) liver microsomes. *A* and *B* represent the GC responses to 15 and 100 ng PBBs injections, respectively.

of binding was given additional credence by the observations of Safe et al. (*31*) and Kohli and Safe (*32*) that 4,4′-dibromobiphenyl could be metabolized via an epoxide intermediate.

The experiments reported here were performed with one serious constraint, namely, that the composition of the ^{14}C-PBBs was far simpler than that of the FireMaster PBB mixture which contaminated much of the Michigan food chain. The gas chromatographic profiles of these two mixtures are shown in Figures 1 and 2. Unfortunately, the two congeners (peaks 1 and 3) found to be metabolized *in vitro* by rat liver microsomes, as well as most other congeners, were virtually absent from the ^{14}C-PBBs, and by virtue of their demonstrated ability to be metabolized, peaks 1 and 3 would be the most likely candidates to be metabolized into DNA-binding derivatives. However, these are not available in radioactive form, and these experiments were done with the only available radioactive PBBs. The ^{14}C-PBB mixture comprises almost exclusively 2,4,5,2′,4′,5′-hexabromobiphenyl (peak 4) and 2,3,4,5,2′,4′,5′-heptabromobiphenyl (peak 8), the two major constituents of the FireMaster mixture of PBBs, and conclusions based on the experiments reported here can only be drawn with regard to these two congeners.

The results demonstrate that trace quantities of ^{14}C-PBBs (less than 0.05% of the substrate added) were associated with microsomal macromolecules following metabolic activation by microsomal enzymes and subsequent exhaustive organic extractions. Whether this radioactivity originated in the two major components or in the other trace components cannot be ascertained. Most of the other FireMaster components can be seen in this ^{14}C-mixture when extremely large quantities are analyzed by gas chromatography (not shown), and one or more of these could be responsible for this low level of binding. Regardless of the nature of the bound compound(s) only a very small amount of binding could be detected, despite the fact that these incubations were performed under nearly the identical conditions as were used to show the metabolism of PBB peaks 1 and 3 and the extremely rapid metabolism of 2,2′-dibromobiphenyl.

Due to the nature of the organic extraction procedure it was very difficult to be confident that all noncovalently bound radioactivity was being extracted. The microsomal pellets remained compact despite the vigorous addition of the ethanol and repeated mixing of the contents. However, the apparent inducibility of the microsomal capacity to catalyze binding supports the conclusion that the binding was covalent, because if the radioactivity were simply being trapped one would expect the same amount of radioactivity to be found in each pellet regardless of the presence of NADPH or the type of microsomes. The greatest binding was seen in microsomes induced by PB or PBBs, and MC induction also increased the binding. This finding is in rough agreement with the results which showed that microsomes induced by PB or PBBs could metabolize certain PBB congeners, while control or MC-induced microsomes were metabolically inactive. The binding to macromolecules is presumed to be to protein, since most of the RNA is known to be removed by the washing procedure used in preparing the microsomes (*16*).

While it appears that a small amount of binding to protein can occur, no binding of ^{14}C-PBBs to DNA could be detected when DNA was included in the reaction mixtures. Denatured DNA was used in these experiments, because King et al. (*33*) had shown denaturation to increase the covalent binding of benzo[a]pyrene 2.5-fold. The type of microsomes used had no effects on the results; no binding was seen when control or MC-PB-, or PBB-induced microsomes were used. It can therefore be concluded that the two major components of PBBs (FireMaster), which together comprise 83% by weight of this mixture, are not metabolically activated into electrophilic DNA-binding metabolites. Whether the remaining PBB components can be bioactivated into DNA binding metabolites remains to be determined. Kimbrough et al. (*34*) have obtained evidence that PBBs cause neoplastic lesions in rat liver, so this possibility must be seriously considered.

The experiments with ^{3}H-benzo[a]pyrene served as a positive control for the analogous experiments with PBBs. Despite the fact that the observed extent of benzo[a]pyrene metabolite binding to DNA was somewhat lower than has been reported in the literature (*18, 33, 35*), these incubations demonstrated that the negative results obtained with ^{14}C-PBBs and DNA under the same conditions were not due to some error or fault in the experimental design.

Benzo[a]pyrene is a well characterized planar aromatic compound which is activated by microsomal drug metabolizing enzymes into a diol epoxide intermediate capable of binding to DNA (*36*). The DNA incubations with ^{3}H-benzo[a]pyrene, in addition to serving as a positive control for the analogous PBB-binding experiment, demonstrate that the induction of microsomal drug metabolizing enzymes by PBBs can greatly enhance (by sixfold) the amount of benzo[a]pyrene metabolites covalently binding to DNA. The increased binding catalyzed by MC-induced microsomes, and the small effect of PB-induced microsomes, confirms the results of

Gurtoo and Bejba (*35*).

The enhanced binding seen following PBBs induction of microsomes suggests that exposure to PBBs may increase the mutagenic and carcinogenic potentials of planar aromatic hydrocarbons administered secondarily. While PCBs have been shown to decrease the carcinogenic potentials of 3'-methyl-4-dimethylaminoazobenzene, *N*-2-fluorenylacetamide, and diethylnitrosamine (*37*), they increase the carcinogenicity of α- and β-1,2,3,4,5,6-hexachlorocyclohexane (*26*). Direct studies on the effects of PBBs on the carcinogenicity of any secondary agents have not been reported.

This investigation has determined only the extent of binding of the two major PBB components to protein and DNA; due to the unavailability of the other radioactive congeners. It is hoped that the congeners known to be metabolized will be available in the future in radioactive form so that their binding to protein, RNA, and DNA, can be determined.

The authors gratefully acknowledge the expert technical assistance of Poonsin Olson and Debra Metcalf. Michigan Agricultural Experiment Station Journal Article Number 8395.

This research was supported in part by National Institutes of Health (Grant number GM 21321).

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